

6/PT5

DETECTING TELOMERASE ACTIVITY

FIELD OF THE INVENTION

The present invention relates to a method for detecting telomerase activity in a sample, and to the use of a solid phase for detecting telomerase activity in a sample. The invention also relates to a kit for detecting telomerase activity in a sample.

BACKGROUND TO THE INVENTION

Telomeres

Telomeres are the specialised ends of all eukaryotic chromosomes that serves as protective caps preventing the chromosomes from fusing together and causing DNA rearrangements that can lead to karyotypic changes and genomic instability (Müller, 1938; McClintock, 1941). The term "telomere" was coined by Müller from the Greek words for "end" (telos) and "part" (meros). The regulatory role for telomeres in cell proliferation was postulated when it was realised that the ends of linear DNA molecules could not be replicated by any known DNA polymerases (Watson, 1972; Olovnikov, 1973). Olovnikov (1973) proposed that loss of telomeric DNA would occur with cell division and ultimately cause loss of genes essential for growth. It has later been shown that telomeres are in a dynamic state of equilibrium between shortening and stabilisation of lengthening (Greider and Blackburn, 1987; Morin, 1989).

Figure 1 presents a model describing the inverse relationship between telomere length and replicative age. As telomeres shorten they will reach a critical length, which signals cell cycle arrest and initiates a senescence program. This point (M1) represents the limit of replicative lifespan, initially described by Hayflick (1965) for human cultured fibroblasts. This property has later been documented for a variety of other cell types growing *in vitro* or *in vivo* (reviewed by Harley, 1995). If a cell bypasses M1 (Hayflick limit), for example by oncogenic transformation, then its telomeres continue to shorten until a second point is reached (M2), termed crisis, where massive cell death occurs, perhaps because of chromosome inviability due to impaired telomere function. For cells to emerge from this crisis, activation of the telomere maintenance machinery, including telomerase, is needed so that telomere length and

structure can be restored and maintained. The cells that emerge from M2 can divide indefinitely. Harley (1991) equated this loss of telomeric DNA with a clock that monitors the number of times a cell has divided and ultimately limits the process of division.

In nearly all eukaryotes, telomeres are complexes of G rich simple repeated DNA and of proteins that specifically bind to it. Table A shows some eukaryotic organisms in which telomerase activity has been identified and the RNA component of telomerase identified and cloned (reviewed by Greider, 1996). The telomere sequence of each organism is shown in 5'→3' direction, as is the sequence of the template region of the telomerase RNA. Size (nt) indicates the length of the RNAs in nucleotides. Human telomeres can contain up to 20 kb of tandem repeats of the hexamer TTAGGG (Moyzis *et al.*, 1988).

Table A

Telomerase RNA components			
Organism	Telomere sequence	RNA template sequence	Size (nt)
<i>Tetrahymena</i>	TTGGGG	CAACCCCAA	159
<i>Euplotes</i>	TTTTGGGG	CAAAACCCCAAAACC	190
<i>Oxytricha</i>	TTTTGGGG	CAAAACCCCAAAACC	190
Human	TTAGGG	CUAACCCUAAC	450
Mouse	TTAGGG	CCUAACCCU	450
<i>Saccharomyces cerevisiae</i>	(TG) ₁₋₃	CACCACACCCACACAC	~ 1300
<i>Kluyveromyces lactis</i>	TTTGATTAGGTATG- TGGTGTACGGA	UCAAAUCCGUACACCAC- AUACCUAUAUCAA	~1300

The end-replication problem

Conventional DNA polymerases can not start DNA synthesis *de novo* and can only replicate DNA in the 5'→3' direction. Replication of the eukaryotic chromosomal DNA molecules is typically primed by an 8- to 12-base stretch of RNA. This mechanism suffices for all but the very ends of the chromosome. Removal of the terminal RNA primer is expected to generate a small gap at the 5' ends of newly replicated lagging strands that can not be repaired by a conventional DNA polymerase. Hence, in the absence of a specialised replication mechanism, a given DNA end is expected to lose 8-12 basepairs in every other S phase (Zakian, 1997). The rate of DNA loss will be even faster if there is degradation or if the terminal RNA primer

is not laid down at the very end of the DNA molecule; and actually telomere shortening has been shown to be in the range of 50 to 100 base pairs per cell generation (Harley *et al.*, 1990; Counter *et al.*, 1992; Vaziri *et al.*, 1994).

Telomerase

There are multiple solutions to the problem of replication of DNA ends. For example, protein priming, terminal hairpins, and/or recombination are used for replication of certain viral and plasmid genomes in both prokaryotes and eukaryotes. However, most eukaryotes from single-celled organisms to higher plants and animals, including humans (reviewed in Greider, 1996), employ telomerase for replicating the chromosome ends. Telomerase is a ribonucleoprotein (Greider and Blackburn, 1987; Morin, 1989) whose RNA and protein components are both essential for the synthesis of telomeric DNA. Telomerase RNA contains an 8- to 30-base segment that serves as template (Table A) for elongation of the 3' overhang that remains after removal of the terminal RNA primer. Telomerase generates tandem repeats of the short sequence encoded by telomerase RNA as shown in Figure 2 (associated protein components are not shown). In (a) the 3' end of the chromosome aligns near the 3' end of the template region of the RNA. The entire template region is complementary to ~1.5 repeats of the human telomerase sequence (Table A). In (b) telomere extension proceeds to the 5' end of the template region. In (c) telomerase can translocate to begin another round of telomere synthesis (adapted from Harley and Villenponteau, 1995). After telomerase elongation of the 3' strand, a conventional DNA polymerase probably synthesises the complementary strand.

Telomerase activity was first characterised biochemically for *Tetrahymena*, a ciliated protozoa, by Greider and Blackburn in 1985. As the structures of all characterised telomeres are very similar, Greider and Blackburn (1985) proposed that there has to be a common mode of telomere replication in all eukaryotes. Four years later, this proposal was supported when a very similar biochemical activity was identified in the immortal human HeLa cell line (Morin, 1989). Cloning of the RNA component of *Tetrahymena* telomerase (Greider and Blackburn, 1989) clarified several aspects of the mechanism of action of this novel DNA polymerase. The predicted template region of the *Tetrahymena* RNA component is complementary to 1.5 repeats of the telomere sequence (Table A), a result that was confirmed by the demonstration

that mutations in this region led to the predicted alterations in telomere sequence when the mutated RNA was overexpressed *in vivo* (Yu *et al.*, 1990).

Many properties of the *Tetrahymena* enzyme are shared by enzymes isolated from other sources, which makes this a valuable model organism. Telomerase preferentially binds to and elongates telomeric sequence primers over nontelomeric sequences. However, telomere-like sequences other than the cognate telomere repeats will serve as efficient primers (Greider and Blackburn, 1987). Thus telomerase extracted from *Tetrahymena* will elongate primers consisting of the telomere repeat from organisms as different as ciliates, humans, plants and yeast. This property of telomerase parallels the ability of telomeres from different organisms to function in yeast (Szostak and Blackburn, 1982; Pluta *et al.*, 1984). The ability of telomerase to preferentially elongate telomeric primers without specificity for an unique sequence suggests that telomerase has a general affinity for G-rich oligonucleotide sequences (Lee and Blackburn, 1993; Morin, 1991; Harrington and Greider, 1991; Collins and Greider, 1993), which differ from the high-affinity sequence-specific recognition of many DNA and RNA binding proteins.

Expression of telomerase

Telomerase activity has been detected at low level in normal peripheral blood cells and in normal bone marrow (Counter *et al.*, 1995; Broccoli *et al.*, 1995; Hiyama, K. *et al.*, 1995; Weng *et al.*, 1996; Hohaus *et al.*, 1997). Subsequent studies have more clearly defined the cellular source. Telomerase is not detected in mature neutrophils but B- and T-lymphocytes have activity. Unfractionated mononuclear cells from normal bone marrow also express telomerase activity, usually at the same level as peripheral blood mononuclear cells. Fractionation of bone marrow cells has revealed that a low level of activity is present in stem cells (CD38⁺, CD38⁻), and that the activity increases in more mature precursor cells (CD34⁺/CD71⁺/CD38⁺) and declines again in maturing CD34⁺ cells (Engelhardt *et al.*, 1997).

Testes and ovaries have also been shown to express telomerase activity (Kim *et al.*, 1994; Wright *et al.*, 1996). It is therefore reason to believe that stem cells generally will show some telomerase activity like demonstrated for the basal cells and stem cells of the skin (Taylor *et al.*, 1996; Härle-Bachor *et al.*, 1996; Yasumoto *et al.*, 1996; Ramirez *et al.*, 1997), intestinal

crypt cells (Hiyama, E. *et al.*, 1996a), endometrium in premenopausal women (Kyo *et al.*, 1997; Brien *et al.*, 1997) and proliferating endothelial cells (Hsiao *et al.*, 1997). Telomerase activity is repressed when inducing differentiation of immortal cells (Holt *et al.*, 1996b; Sharma *et al.*, 1995; Bestilny *et al.*, 1996; Xu *et al.*, 1996; Asai *et al.*, 1998). This leads to the working hypothesis that there are at least two pathways for the repression of telomerase activity (Holt *et al.*, 1996a). One pathway represses telomerase in most somatic tissues late in development (Wright *et al.*, 1996). These somatic cells, representing the vast majority of the cells in the body, do not express telomerase activity regardless of their proliferative state. The remaining cells in the body are telomerase competent. The second pathway reversibly regulates the telomerase activity so that it is present during cell proliferation and repressed during periods of quiescence. This model explains a variety of otherwise confusing results; telomerase activity is found in testis and ovary, while it is absent in sperm and oocytes, present in early embryogenesis, but decreases during development of the fetus and is absent in most tissues in the new-born baby (Wright *et al.*, 1996). The low activity in the most primitive stem cells compared to the more mature cells (Counter *et al.*, 1995; Hiyama K. *et al.*, 1995) is another phenomenon that can be explained from the second pathway. However, most of these cells are in a quiescent state, and their proliferating progeny express higher levels of telomerase activity (Wright *et al.*, 1996).

Telomere length and telomerase activity were also found to be closely regulated in the course of B cell differentiation (Weng *et al.*, 1997b). In contrast to previously characterised models of telomere shortening during somatic cell division, telomeres are significantly longer in germinal centre B cells than in either precursor naive or descendant memory B cells. Telomerase activity was markedly increased in germinal centre B cells relative to other B cell populations, correlating with increased telomere length (Weng *et al.*, 1997b). High levels of telomerase activity are induced in naive and memory B cells by *in vitro* activation. These results suggest that telomerase may play a critical role in immune responses by elongating telomeres and thus preserving the replicative lifespan of germinal centre and progeny memory B cells.

Expression of the RNA component of telomerase

It has been shown that the human telomerase RNA component (hTR) is expressed at detectable levels in normal somatic cells, even though most of these cells have no detectable telomerase activity (Feng *et al.*, 1995). However, the parallels observed between up-regulated telomerase activity and up-regulated hTR expression, both during T cell development and during *in vitro* T cell activation, suggest that regulation of hTR levels may contribute to the overall regulation of telomerase activity in normal somatic cells such as lymphocytes (Weng *et al.*, 1997a). Expression of hTR may be necessary but not sufficient for telomerase activity, and the absence of telomerase activity in resting lymphocytes with detectable levels of hTR may reflect the failure to express sufficient levels of one or more of the other telomerase components or the presence of a dominant mechanism to repress telomerase in these cells (Weng *et al.*, 1997a).

The catalytic subunit and other telomerase associated proteins

All immortalised human cell lines examined to date contain either telomerase activity or evidence of an alternative mechanism for lengthening of telomeres (Bryan *et al.*, 1997). This represents very strong circumstantial evidence for the importance of telomere maintenance in immortalisation. The catalytic subunit of telomerase, hTERT (human telomerase reverse transcriptase, initially abbreviated hTRT, hEST2, hTCS1 or TP2), was recently identified and cloned (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997; Kilian *et al.*, 1997). The gene coding for hTERT is present in a single copy in the human genome but is expressed in a complex splicing pattern. In addition to transcription control of the hTERT gene, alternative splicing of the telomerase catalytic subunit may be important for the regulation of telomerase activity and may give rise to proteins with different biochemical functions (Kilian *et al.*, 1997; Ulaner *et al.*, 1998).

Expression of hTERT positively correlates with the known telomerase status of tissues and cell lines and occurs preferentially in cell lines *in vitro* after the cells have undergone crisis, in normal tissues with a significant stem cell component and in a range of tumours (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997; Kilian *et al.*, 1997; Kolquist *et al.*, 1998; Kanaya *et al.*, 1998). These findings define an important component of the human telomerase complex and implicate its expression as the major determinant of the distribution of telomerase activity in

mammalian cells. The importance of hTERT was demonstrated when amino acid changes in conserved telomerase specific and reverse transcriptase (RT) motifs reduce or abolish telomerase activity (Weinrich *et al.*, 1997). Furthermore, transfection experiments indicate that hTERT is the limiting factor for the activation of telomerase in normal diploid cells (Weinrich *et al.*, 1997; Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). It was recently shown that hTERT mRNA is present in specific subsets of some normal, telomerase negative tissues thought to have long term proliferative capacity, but the level may not signify a level of telomerase that is above the threshold required for telomere maintenance or elongation (Kolquist *et al.*, 1998).

While the RT-like telomerase proteins are involved in catalysis, additional proteins such as telomere- and telomerase associated proteins are required for full telomerase assembly, function and regulation. Two protein subunits have been identified in *Tetrahymena* (p80 and p95) that appear to be associated with the telomerase complex but not being the catalytic subunit (Collins *et al.*, 1995). This led to the identification of mammalian homologues, TP1 (Harrington *et al.*, 1997; Nakayama *et al.*, 1997), which RNA transcript does not correlate with the telomerase activity. Cloning and characterisation of a human telomeric-repeat binding factor revealed that human telomeres form a specialised nucleoprotein complex (Chong *et al.*, 1995). Recently it was reported that this protein, TRF1, is involved in regulating the telomere length (van Steensel and de Lange, 1997).

Expression of telomerase in cancer cells

Presence of telomerase activity in immortal human cell populations and malignant tumours and its general absence prior to this stage, has led to the suggestions that cellular immortalisation and telomerase reactivation are essential for the progression and clinical lethality of most human tumours, but not for their formation (Kim *et al.*, 1994; Edington *et al.*, 1995). In many cases of cancer, the inactivation of tumour suppressors such as p53 and pRb, which allows cells to overcome M1 and have an extended lifespan (Figure 1.), directly leads to the genomic instability that contributes to the accumulation of additional mutations (Harley, 1991, 1995). Maintenance of telomeres at very short lengths, as is the case for many human tumours, may be sufficient to stabilise chromosome ends enough for cell division. Yet,

this stabilisation may not totally eliminate the potential for increased chromosomal recombination, which could contribute to genomic instability and tumour progression.

The method used for detection of telomerase activity is called TRAP assay (telomeric repeat amplification protocol) (Kim *et al.*, 1994). The cells are lysed in a way that keeps the telomerase activity intact. After enzymatic elongation of an added synthetic oligonucleotide, the specific telomeric product is amplified by PCR for detection. This method has simplified the work in the telomerase field a lot. To date nearly all types of human neoplasia have been screened for the presence of the enzyme, and 759 of 895 (i.e. 85%) of malignant tumours tested have been shown to express telomerase activity (Shay and Bacchetti, 1997).

WO97/15687 describes a two reaction protocol for detecting telomerase activity. In the first reaction telomerase substrate extension products are formed from a telomerase substrate, while the second reaction involves replication of the extension products and/or amplification of signal generated by a bound probe.

Expression of hTR in telomerase negative somatic cells as well as telomerase positive malignant cells, should indicate that the presence of the RNA component is not the only factor required for enzymatic activity. This is in accordance with the findings that the levels of hTR is not affected when telomerase activity is decreased during experimentally induced differentiation of tumour cells *in vitro* (Feng *et al.*, 1995), and that the level of hTR in a variety of different tumours is not shown to predict the level of telomerase activity in the tumour (Avilion *et al.*, 1996).

In contrast, hTERT correlates with telomerase activity (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997; Kilian *et al.*, 1997; Kolquist *et al.*, 1998; Kanaya *et al.*, 1998). Expression of hTERT is up-regulated concomitant with the activation of telomerase during immortalisation of cultured cells and down-regulated during *in vitro* cellular differentiation (Meyerson *et al.*, 1997). These results suggest that induction of hTERT mRNA expression is required for telomerase activity during cellular immortalisation and tumour progression.

Telomerase as a diagnostic and possible prognostic marker

Given the association between telomere length and telomerase activity with cancer and senescence, it is reasonable to analyse and quantify telomerase activity in tumour cells. This

may be a diagnostic and prognostic marker and itself a target for therapy. The present data suggest that telomerase may be the most specific and universal cancer marker known to date. Furthermore, a number of studies have shown that telomerase expression correlates with

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clinical outcome in some cancers (reviewed in Bacchetti and Counter, 1995; Shay and Wright, 1996; Shay and Bacchetti, 1997; Le *et al.*, 1998; Yoshida *et al.*, 1998), which means that telomerase can be used as a prognostic marker as well as a diagnostic marker in these kinds of cancer. Generally, the levels of telomerase activity increases with cancer disease severity (Counter *et al.*, 1995; Hiyama E. *et al.*, 1995a, 1996a), but there is a substantial variability even among tumours of the same type and stage. The data collected indicate that telomerase is a prevalent and specific tumour marker (Bacchetti and Counter, 1995; Leber and Bacchetti, 1996; Shay and Wright, 1996; Shay and Bacchetti, 1997). The presence of telomerase activity can indicate presence of either normal telomerase-competent cells or cancer cells.

Correspondingly, the absence of telomerase-activity can indicate either presence of telomerase-silent normal cells or the quiescent state of telomerase-competent cells. Therefore, the prognostic value of levels of telomerase activity is limited unless careful control experiments are done. This can be illustrated by the fact that certain haemotopoietic cells show low levels of telomerase activity, which complicates the detection of circulating cancer cells in blood and bone marrow.

High levels of enzyme correlate with poor clinical outcome in neuroblastoma (Hiyama E. *et al.*, 1995a). Interestingly, patients with metastatic IV-S neuroblastoma, a disease stage known to have a high frequency of natural regression, showed in general no or low telomerase activity. In fact, those with no tumour telomerase expression experienced spontaneous regression of the tumours. Also high levels of the telomerase RNA component have been shown to have a statistically correlation with poor clinical outcome in neuroblastoma patients (Kim, 1997). Clinical outcome and several prognostic indicators of breast cancer have been shown to have a statistically significant correlation with the level of telomerase activity (Hiyama E. *et al.*, 1996b; Kim, 1997). Similarly, telomerase activity was found in 85% of gastric tumours and the majority of the negative cases was early-stage (Hiyama E. *et al.*, 1995b; Tahara *et al.*, 1996). Chadeneau *et al.* (1995) showed that telomerase activity could not be detected in adenomatous polyps of the colon whereas it could be detected in colorectal carcinomas. Thus for carcinomas and neuroblastomas there may be a prognostic value in knowing the telomerase status.

Telomerase expression can be a useful marker for predicting outcome of disease. In addition, such a novel marker might provide new information for determining appropriate treatment for a cancer patient. Recently, it was shown that telomerase activity was inhibited by cisplatin treatment in human testicular cancer (Burger *et al.*, 1997), and that the telomerase activity in different malignant cells was inhibited when introducing retroviruses expressing RNA complementary to the template region of hTR (Bisoffi *et al.*, 1998). The value of a prognostic marker will be most evident where there are clearly defined treatment options that are dependent upon the aggressiveness of the tumour in question. Thus, a telomerase-based prognostic assay could for example differentiate node-negative breast cancer patients either by screening of the primary tumour for telomerase expression level or screening of circulating cancer cells (micrometastasis). The latter patients are likely to experience tumour recurrence and metastatic disease and will benefit from more an aggressive therapy. Telomerase detection may be a prognostic marker during treatment of cancer since there is evidence for that telomerase activity can be turned off by inducing cancer cells to differentiate (Sharma *et al.*, 1995; Bestilny *et al.*, 1996; Xu *et al.*, 1996).

Micrometastasis

During routine screening, for example by mammography, suspicious lumps can be detected. By obtaining a sample of this lump, the pathologist can readily diagnose whether the cells are malignant or not. However, the fate of the cancer patient is far more difficult to determine. It depends on a cellular event that is very hard to observe directly; metastasis, the spread of malignant cells throughout the body that induces secondary tumours. When a cancer treatment fails, metastasis is usually the primary cause of death. If a primary tumour is detected early and removed before metastasis occurs, the cancer will be eradicated. On the other hand, if even microscopic metastases (micrometastasis) or secondary tumours are already present at the time of diagnosis, then the prognosis is grave and tougher treatment is needed. Left untreated, these metastasis will normally grow and prove fatal. Therefore, an early detection of cancer is of major importance, and the detection of metastasis and even micrometastasis is important for choosing the right treatment regimen.

The problem of detecting cancer cells at an early stage and micrometastasis in blood or bone marrow, is the problem of detecting rare cancer cells among a huge excess of normal cells (Pelkey *et al.*, 1996). The detection system needed for this can be based on:

- locating cells at a site they should not be present
- a component present in cancer cells but not in normal cells
- a combination of the two above

The technology presently used is based on microscopic detection of cancer cells. This can be made easier by selective staining of cancer cells made possible by cells present at an abnormal location. An example of this is staining of cytokeratin, an epithelial cell-specific protein also found in carcinoma cells circulating in blood or bone marrow. Another possibility is to isolate mRNA and do an amplification of genes expressed more or less specifically in cancer cells. The latter approach is very difficult since even tissue-specific genes is normally present at a very low level in so called "non-expressing cells". There can be a big difference in expression, for example 10 000 copies per cancer cell and 1 mRNA per normal cell. However, this is more than outweighed by the ratio of rare cancer cells to normal cells, for example 1 per million (e.g. a mRNA ratio of 10 000 mRNA copies in cancer cells and 1 million in normal cells). This is of course made almost impossible when the cancer cell is present in its "normal" environment, like trying to detect colon carcinoma cells in faeces among the excess of normal colon cells.

Both the microscopic approach and the mRNA approach can be made feasible by an enrichment of cancer cells, either by depletion of normal cells or positive selection of cancer cells (Hardingham *et al.*, 1993, 1995; Eaton *et al.*, 1997; Naume *et al.*, 1997; Lycke *et al.*, 1997). However, all these strategies are more or less based on the ability to detect the cancer cell at an "abnormal" location, for example epithelial derived carcinoma cells in blood or bone marrow.

The present invention aims to overcome the disadvantages of the prior art.

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SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method for cancer diagnosis or prognosis which comprises:

(a) treating a sample from a human or animal subject with a solid phase under conditions to bind telomerase to the solid phase;

(b) separating the solid phase from the treated sample to form a test sample which is optionally treated to elute bound telomerase from the solid phase; and

(c) assaying the test sample for telomerase activity,

wherein detection of telomerase activity in the sample is indicative of cancer in the subject.

In a further aspect the present invention provides use of a solid phase for detecting telomerase activity in a sample by treating the sample with the solid phase so as to bind the telomerase thereto and assaying the solid phase for telomerase activity.

In a further aspect the present invention provides use of a kit for detecting telomerase activity, wherein the kit comprises a solid phase for binding telomerase, and one or more components for assaying for telomerase activity, and wherein the solid phase is used to bind telomerase.

In a further aspect, the present invention provides a kit for detecting telomerase activity, comprising a solid phase for binding telomerase and one or more components for assaying for telomerase activity, wherein the solid phase comprises an affinant for binding target whole cells.

In a further aspect, the present invention a kit for detecting telomerase activity, comprising a solid phase for binding telomerase and one or more components for assaying for telomerase activity, which further comprises a second solid phase for binding target whole cells.

In a further aspect the present invention provides use of a kit as described herein, for the detection of cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a graph of telomere length against replicative age for various cell types.

FIGURE 2 shows a representation of the predicted mechanism of human telomerase.

FIGURE 3 shows a polyacrylamide gel as described in Example 1 illustrating the detection limit for the telomerase repeat assay.

FIGURE 4 shows a polyacrylamide gel as described in Example 2 illustrating the isolation of telomerase activity with magnetic beads.

FIGURE 5 shows a polyacrylamide gel as described in Example 3 illustrating the isolation of telomerase activity using Dynabeads[®] DNA DIRECT.

FIGURE 6 shows a polyacrylamide gel as described in Example 4 illustrating the isolation of telomerase with increasing quantities of epoxide beads.

FIGURE 7 shows a polyacrylamide gel as described in Example 5 illustrating a test for the presence of genomic DNA in the remaining lysate fractions after bead-based telomerase isolation.

FIGURE 8 shows a SDS-PAGE gel as described in Example 6 illustrating the binding of proteins to polymer magnetic beads.

FIGURE 9 shows a polyacrylamide gel as described in Example 7 illustrating the sensitivity and reliability of the telomerase isolation method.

FIGURE 10 shows a polyacrylamide gel as described in Example 8 illustrating the enrichment of circulating carcinoma cells in blood with subsequent detection of telomerase activity.

FIGURE 11 shows a polyacrylamide gel as described in Example 9 illustrating telomerase activity in micrometastatic cells in pleura from a breast cancer patient.

FIGURE 12 shows a flowchart illustrating the combination of the telomerase isolation with the TRAP assay and mRNA isolation from the lysate for detection of micrometastatic cells.

FIGURE 13 shows a flowchart illustrating a method for detecting telomerase activity in colonic cells isolated from faeces.

The invention will now be described in further detail, by way of example only.

The method used for determining telomerase activity is called TRAP assay (telomeric repeat assay protocol) (Kim et al. 1994). The cells of interest are lysed in a way that keeps the telomerase activity intact. A synthetic oligonucleotide is added to the lysate, and the mixture

[illegible]

is incubated at 37° C to allow for the telomerase to use this oligo for elongation. This specific telomeric product is amplified by PCR for detection. However, this method still involves the analysis of ³²P -labelled reaction products by polyacrylamide gel electrophoresis (Kim et al. 1994, Piatyszek et al. 1995). Modifications such as introduction of an internal standard (Wright et al. 1995) have improved the reliability of the assay. A further improvement is done by combining the TRAP assay with a scintillation proximity assay technology (SPA) (Savoysky et al. 1996). This increases the speed of the assay by omitting the electrophoresis step. Whereas, for the TRAP-PAGE, the detection limit is about 100 cells per assay, in the case of TRAP-SPA, the telomerase activity could clearly be detected with as few as 10 cells. Improved primer design and development of a semi-quantitative assay has also increased the reliability of the assay (Kim and Wu 1997).

In general, the current gold standard for most cancer diagnosis is histopathology, which in most cases provides an accurate assessment of the suspected tissue biopsies. Therefore, the most significant clinical utility of a telomerase assay could be in the diagnosis of cancer through obtainable body fluids such as blood, bone marrow, urine, sputum and materials derived from various washes. Fine needle biopsies, cervical smears and analysis of cells in the faeces might also be useful for improved early diagnosis of primary cancer.

Method for isolation of telomerase enzyme complex

Initial experiments using magnetic beads to isolate cells prior to running a TRAP assay gave confusing results. The magnetically isolated cells were lysed in the lysis buffer included in the TRAPeze kit (Oncor Inc., USA), the magnetic beads removed and the telomeric repeat assay protocol performed using the lysate. The results showed either no activity or very weak activity even in cell lines known to be strongly positive. Surprisingly, the telomerase activity follows the magnetic beads after cell lysis and with enough beads present all activity can be removed from the lysate (Example 2, 3, 4, 7). We have developed this into a simple and rapid method for isolating telomerase complexes from crude lysates. The TRAP assay has been shown to be prone to inhibition by some lysates - this is especially true if too much material is analysed (Broccoli et al. 1995, Norrback 1996). For each extract initial TRAP reactions must be performed to identify conditions under which the TRAP reaction are not inhibited. This has usually been done by measuring the protein content in the lysate and using samples containing

0.1-1.0 µg per assay since 2.5 µg to 25 µg has been shown to result in inhibition (Broccoli et al. 1995). Including an internal control will reveal any inhibition (Wright et al. 1995). It has been shown that several samples tested to be negative were actually false negatives due to inhibition of the TRAP assay (Sugino et al. 1997). By isolating the telomerase complexes we are able to avoid any inhibition and much larger samples can be screened for telomerase activity. This is especially useful if the telomerase-positive cells represent a minor fraction of the sample for example stem cells in blood, bone marrow or tissues or cancer cells metastasis in lymph nodes or circulating micrometastasis.

We have been using different kinds of magnetic Dynabeads®, Antibody coated beads of 2,8 (Example 2, 8 and 9) or 4,5 µm size, uncoated magnetic beads of the same sizes and Dynabeads® DNA DIRECT (Example 3 and 4), and all bead types tested so far isolate the telomerase complexes. The fact that telomerase works on chromosomal DNA ends made it natural to put forward a hypothesis that telomerase was bound indirectly to the magnetic beads via chromosomal DNA. This hypothesis was supported when it was shown that beads developed for isolation of DNA also could be used for capture of telomerase (Example 3). To verify this assumption, it had to be proven that DNA was present in the bead fraction after isolation of telomerase, but the hypothesis had to be rejected since DNA remained in the lysate after telomerase isolation (Example 4).

The magnetic particles used in this study, except from those developed for DNA isolation, were coated with aromatic epoxide monomers and had a hydrophilic surface, which in general will immobilise biomolecules. This hydrophilic surface has an advantage over hydrophobic surfaces, as denaturation of the captured enzyme will be avoided and thereby reduce loss of enzymatic activity over time. We do not want to be limited by any explanation but a likely explanation that is telomerase isolated by the magnetic beads through non-specific binding of the telomerase complex along with other proteins, protein complexes and RNA-protein complexes (Example 6). We assume that this method for isolating telomerase complexes also will be useful for isolating any kind of proteins, RNA or complexes, and that this can be designed by changing the surface properties of the solid phase. The lysis buffer in the TRAPeze kit includes 0,5 % CHAPS, 1 mM EGTA, 5 mM β-mercaptoethanol and 0.1 mM

benzamidine. This results in cell lysis but there is no strong denaturing component and RNA and proteins complexes are kept intact.

Experiments performed (Examples 2-9) show that the telomerase complexes attached to the magnetic beads remain enzymatically active after removal of the cell lysate. This implies that the beads with the captured and purified telomerase complexes can be added directly to the TRAP mix for enzymatic elongation of the telomere with subsequent PCR amplification of the telomeric product. The isolated telomerase can also be used for any other kind of method for testing telomerase activity.

The findings that the telomerase activity follows the magnetic beads simplifies greatly the telomerase assay. The main advantage of the method invented is the separation of the telomerase complex from the lysate, which may contain inhibitors of the assay causing false negatives. Too much cell extract added to the TRAP assay can also inhibit the telomerase activity (Broccoli *et al.*, 1995). Negative TRAP assays need careful interpretation as demonstrated when phenol-chloroform extraction, after the telomerase extension reaction, of previously telomerase negative samples revealed presence of PCR inhibitors in 30% of these samples (Sugino *et al.*, 1997). Use of magnetic beads coated with antibodies towards epithelial cells (Example 2 and 8), non-coated epoxide beads (Example 4) and magnetic beads developed for isolation of DNA (Example 3) for capture telomerase is an advantage when performing the TRAP assay for detection of enzymatic activity. Lysis buffer can be separated from the complex of telomerase and beads and in this way reduce the probability for false negative results.

The complex of beads and telomerase was separated from the cell lysate using a magnet. In this way interfering agents were removed along with the lysate, before the complex of telomerase and beads was resuspended in fresh lysis buffer or dH₂O for telomere synthesis and PCR amplification. A clean bead fraction also leads to an improved reliability of the assay even when working close to the detection limit (Example 7). Removal of most of the contaminating factors reduces the probability of obtaining false negative results, and hence increases the reliability of the TRAP method. This will presumably also be an advantage where there are problems with false negative tests due to the mucous nature of some tumours

(Duggan *et al.*, 1998). Magnetic beads could be used to isolate telomerase and remove the viscous lysate prior to the enzymatic synthesis.

The same sensitivity without radioactive labelling was achieved with the telomerase isolation method as Kavalier *et al.* (1998) achieved with radioactive labelling using exactly the same primers, buffers and PCR conditions! With a sensitivity of approximately 50 cells in both studies, use of a solid phase for isolation of telomerase will therefore be in preference to radioactive labelling to avoid radiation and the time-consuming exposure of the X-ray film to gain signals strong enough for visualisation.

The beads can still be used to achieve capture of telomerase even when using methods not based on immunomagnetic separation of cancer cells. By adding beads directly to the cell lysate, the telomerase complex can still be isolated and separated from the lysate without centrifugation, which will be more lenient to the functional enzyme. Another advantage using a solid phase like magnetic beads for telomerase capture, is presumably the yield of the isolation procedure.

Solid phase used for telomerase isolation

The solid support may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation, purification etc. These may take the form of particles, sheets, filters, membranes, fibres, capillaries, microtitre strips, tubes, plates, well or biochips etc.

Conveniently the support may be made of glass, silica, latex, cellulose, agarose or any polymeric material. Preferred are materials presenting a high surface area for binding of the telomerase complex and other proteins or protein complexes. Such supports will generally have an irregular surface and may be for example be porous or particulate eg. particles, fibres, webs, sinters or sieves. Particulate materials eg. beads are generally preferred due to their greater binding capacity, particularly polymeric beads.

Conveniently, a particulate solid support used according to the invention will comprise spherical beads. The size of the beads is not critical, but they may for example be of the order

of diameter of at least 1 and preferably at least 2 μm , and have a maximum diameter of preferably not more than 10 and more preferably not more than 6 μm . For example, beads of diameter 2.8 μm and 4.5 μm have been shown to work well.

Monodisperse particles, that is those which are substantially uniform in size eg. size having a diameter standard deviation of less than 25 %, preferably less than 10 % and more preferably less than 5 %, have the advantage that they provide very uniform reproducibility of reaction. Monodisperse polymer particles produced by the technique described in US-A-4336173 are especially suitable.

Non-magnetic polymer beads suitable for use in the method of the invention are available from Dyno particles AS (Lillestrøm, Norway), as well as Seradyn (Indianapolis, US), Qiagen (Germany), Amersham Pharmacia (UK) and others. Magnetic particles suitable for use in the method of the invention are available from Dynal (Oslo, Norway), Seradyn (Indianapolis, US), Scigen (Sittingbourne, UK) and others.

However, to aid manipulation and separation magnetic beads are preferred. The term "magnetic" as used herein means that the support is capable of having a magnetic moment imparted to it when placed in a magnetic field, and thus is displaceable under the action of that field. In other words, a support comprising magnetic particles may readily be removed by magnetic aggregation, which provides a quick, simple and efficient way of separating the particles following the cell and molecular binding steps, and is a far less rigorous method than traditional techniques such as centrifugation which generate shear forces which may disrupt cells, degrade molecules or destroy the biological activity of the isolated component.

Thus, using the method of the invention, the magnetic particles with telomerase complex and other proteins or RNA-protein complexes attached may be removed onto a suitable surface by application of a magnetic field eg. using a permanent magnet. It is usually sufficient to apply a magnet to the side of the vessel containing the sample mixture to aggregate the particles to the wall of the vessel and to pour away the remainder of the sample.

Especially preferred are super-paramagnetic particles for example those described by Sintef in EP-A-106873, as magnetic aggregation and clumping of the particles during the reaction can be avoided, thus ensuring uniform isolation the biological material. The well-known magnetic particles sold by Dynal AS (Oslo, Norway) as DYNABEADS®, are particularly suited to use in the present invention.

Functionalised coated particles for use in the present invention may be prepared by modification of the beads according to US patents 4336173 and 4,459,378 and 4,654,267. Thus, beads, or other supports, may be prepared having different types of functionalised surface, for example positively or negatively charged, hydrophilic or hydrophobic.

Different molecular components exhibit different degrees of non-specific binding to different surfaces and supports and it may be advantageous to "titrate" the amount of solid support (eg. the number of particles) per volume unit, in order to optimise the binding conditions, and determine the optimum support area eg. particle concentration for a given system.

The cell sample, isolated or support-bound cells are lysed to release their components including telomerase complexes. Methods of cell lysis are well known in the art and widely described in the literature and any of the known methods may be used. Different methods may be more appropriate for different sample types and different cells, but to keep the biological activity of the target components it is important to use an appropriate lysis method with appropriate buffers and mechanical lysis.

To carry out the method of the invention, the cells may be isolated by any means like centrifugation of liquid samples, needle biopsies, buccal scrapes, preferentially for the method of the invention cells are enriched by using a solid support and the support-bound cells may conveniently be removed or separated from the remainder of the sample, thereby concentrating or enriching the cells. Thus the cell binding step serves to enrich the cells and/or to concentrate them in a smaller volume than the initial sample. Lysis may then conveniently be achieved by adding an appropriate lysis buffer containing the desired lysis agents or by subjecting the isolated cells to the desired lysis conditions. For example, in the case of simply adding a lysis buffer containing the appropriate lysis agents, the isolated cells may simply be

incubated in the presence of the lysis buffer for a suitable interval to allow for lysis to take place without destroying the biological activity of the target component eg. the telomerase.

Telomerase activity and primary cancer diagnostics

To date nearly all types of human neoplasia have been screened for the presence of telomerase activity and 759 of 895 (i.e. 85%) of the malignant tumours tested were shown to express this enzyme (Shay and Bacchetti, 1997), a result that can be utilised to distinguish malignant from non-malignant tumours (Sommerfeld *et al.*, 1996; Hiyama *et al.*, 1996b; Sugino *et al.*, 1996; Kyo *et al.*, 1997; Murakami *et al.*, 1997; Yoshida *et al.*, 1998).

Several research groups are working hard to find a solution to the problem of detecting exfoliated cancer cells using telomerase as a diagnostic marker, but neither of them have come up with a reliable method yet. False negative results due to inhibitors of the TRAP assay is the main problem in the search for exfoliated cancer cells using telomerase activity as the diagnostic marker (Califano *et al.*, 1996; Müller *et al.*, 1996, 1998; Yoshida *et al.*, 1997a, b; Heine *et al.*, 1998; Kavalier *et al.*, 1998; Sumida *et al.*, 1998). Until recently it has been problematic to detect telomerase activity in exfoliated carcinoma in voided urine due to presence of PCR inhibitors (Müller *et al.*, 1996, 1998; Heine *et al.*, 1998). However, this problem has more or less been overcome by several washings of the exfoliated cells after centrifugation (Yoshida *et al.*, 1997b; Kavalier *et al.*, 1998), but it is a time consuming procedure with an extensive loss of cells.

Recently, Murakami *et al.* (1998) published a rather successful protocol for enrichment of cancer cells from body cavity fluids and uterine washings. The presence of activated lymphocytes might give false positive results, large amounts of blood could interfere with PCR and the proteins in blood cell components might dilute proteins from cancer cells. Inclusion of blood and lipid components had therefore to be minimised during collection of the samples. To increase the cancer cell ratio and remove erythrocytes and lymphocytes, tumour cells were isolated using Nycoprep (Nycomed Pharma AS), a method based on the same principles as Lymphoprep. But it was observed that the centrifugation steps in the enrichment procedure caused inactivation of telomerase and reduced the number of tumour

cells to be collected. These problems were overcome by culturing the cells for 24-48 hours before performing the TRAP assay.

Adding immunomagnetic beads coated with antibodies towards epithelial cells to enrich circulating cancer cells from any body fluid with subsequent washing of the complex of bead and cells, will remove interfering substances like blood, bacteria and other non-cancerous cells from the captured cells. Experiments where immunomagnetic separation techniques for enrichment and detection of viable breast carcinoma cells in bone marrow and peripheral blood have been tested, have revealed a 3 log enrichment of the cancer cells with a recovery of 70% (Borgnes *et al.*, 1996; Naume *et al.*, 1997). With this knowledge there are strong reasons to believe that such an enrichment of cancer cells is much better than the methods based on centrifugation, as described above (Murakami *et al.*, 1998; Yoshida *et al.*, 1997b; Kavalier *et al.*, 1998). As the ribonucleoprotein telomerase complex and the PCR amplification are both very sensitive for interfering agents, removal of possible inhibitors prior to detection of the enzymatic activity of telomerase is an important part of the protocol. This step is simplified by the use of beads with affinity for telomerase utilising the magnetic properties of the beads. Both the complexes of beads with enriched cancer cells and beads with captured telomerase can easily be washed and in this way interfering agents will be removed. Murakami *et al.* (1998) described the problem centrifugation entails on telomerase activity, but by using immunomagnetic isolation of cancer cells and telomerase this problem is avoided; there will be no need for a time consuming step for culturing of cells before performing the TRAP assay because interfering agents are removed before enzymatic elongation of the telomere and PCR amplification.

The clinical relevance of telomerase has been demonstrated in several independent experiments. In one case reported, no tumour was visualised at cytoscopic examination of the urine bladder, while a bladder washing at the same time showed telomerase activity. Repeating the cytoscopic examination of this patient four weeks later revealed presence of a small bladder tumour (Müller *et al.*, 1996). In another case, telomerase activity was detected in the pancreatic juice 19 months before the affected person got the diagnosis of pancreatic cancer using computed tomography for screening (Suehara *et al.*, 1998). These two examples illustrate how proliferating tumours can be revealed by detection of telomerase before they are

big enough for visualisation, and that the enzymatic activity in this way can be used for making a diagnosis at an earlier stage than today.

In general, the current gold standard for most cancer diagnosis is histopathology, which in most cases provides an accurate assessment of the suspected tissue biopsies. Therefore, the most significant clinical utility of a telomerase assay could be in the diagnosis of cancer through obtainable body fluids such as blood, bone marrow, urine, sputum, and materials derived from various washes as demonstrated by isolation and detection of telomerase in pleura (Example 9). Fine needle biopsies, cervical smears and analysis of cells in the faeces might also be useful for improved early diagnosis of primary cancer.

Micrometastasis detection by telomerase activity

Isolation of circulating micrometastasis has been shown to be an important way of proving cancer dispersion. The problem of detecting cancer cells at an early stage and micrometastasis in blood and bone marrow, is the problem of detecting rare cancer cells among a huge excess of normal cells (Pelkey *et al.*, 1996). The main problem when using telomerase activity as a marker for detection of circulating cancer cells is false negative results due to inhibitors of the TRAP assay (Sugino *et al.*, 1996, 1997; Cunningham *et al.*, 1998; Yang *et al.*, 1998). High levels of peripheral blood cell contamination is one reason for false negatives because it would dilute out the telomerase derived from the intended target cell population (Cunningham *et al.*, 1998). A sensitivity of 91.4% and a specificity of 94.2% was achieved when testing the diagnostic value of telomerase activity on pleural effusion (Yang *et al.*, 1998). As Murakami *et al.* (1998), Yang *et al.* (1998) were concerned about the presence of leukocytes in the effusion samples. Contamination of leukocytes might give false positive results that could undermine the diagnostic value of the TRAP assay.

Detection of micrometastasis in blood was demonstrated in Example 8 with immunomagnetic enrichment of cancer cells and telomerase capture in combination with detection of telomerase activity. Several independent experiments showed a sensitivity of 2-3 cancer cells per million normal cells, which should demonstrate both the sensitivity and the reliability of this method. The same protocol was tested on clinical material, which resulted in detection of micrometastatic cells in pleura from breast cancer patients (Figure 11). Telomerase activity was

found mainly in the bead fraction but some activity was also found in the remaining lysate. This result can be explained by presence of more telomerase complexes in the samples tested than the beads were able to capture.

This invention describes a novel method for detection of micrometastasis; circulating cancer cells were enriched following an immunomagnetic isolation protocol (Bosnes *et al.*, 1997), telomerase was isolated and then detected using the TRAP assay (Kim *et al.*, 1994).

Immunomagnetic isolation and TRAP assay are both recognised methods in molecular biology, but the combination of these two methods has never been described earlier. Enrichment of circulating carcinoma and isolation of telomerase in combination with detection of enzymatic activity can be a contribution in the use of telomerase as both a diagnostic and a prognostic marker.

In summary, this invention describes a method for improved telomerase detection and in addition how this method is combined with targeting cells of interest by cell sorting prior to telomerase detection. One example of this is positive selection of circulating carcinoma cells in blood or bone marrow combined with telomerase detection.

There is provided:

A method for telomerase detection by isolating the telomerase complex from crude cell lysates using a solid-phase and preferentially magnetic beads.

A method for telomerase detection in target cells by positive enrichment of target cells or depletion of unwanted cells like telomerase-competent stem cells, or a combination of positive and negative enrichment strategies to target only cells of interest and removing cells interfering with the assay.

A method for telomerase detection by combining telomerase isolation with detection of telomerase subunits like telomerase RNA. This method detects only components integrated in telomerase complexes and will not detect free subunits in the cell.

A simple and reliable method of detecting primary cancer cells in body fluids or faeces by combining cell purification with telomerase complex isolation and telomerase detection.

Example 1: Telomer Repeat Assay Protocol – TRAP Assay

Detection of telomerase activity in clinical samples with rare cells requires a sensitive assay. The aim of this example was to determine the sensitivity of the telomerase repeat assay without any radioactive labelling. To do so, a series of samples with increasing number of cells 40 to 1000 were set up. The telomerase activity - TRAP assay - was measured in each case (Figure 3), following the original TRAPeze protocol (Oncor).

Method

The SW480 cultured cells of epithelial origin, were washed twice in PBS and collected by centrifugation at 600x g for 10 minutes at 4°C.

The supernatant was discarded and the cells were lysed in 20 µl CHAPS lysis buffer and incubated for 25-30 minutes on ice.

For telomerase assay, a mix was prepared:

5 µl	10x TRAP buffer
1 µl	50x dNTP mix
1 µl	TS primer
1 µl	TRAP primer mix
2 Units	Taq polymerase
X µl	Cell lysate
Total volume 50 µl	

The mix was incubated 30 minutes at 30°C for telomerase-mediated extension of the TS-primer.

Amplification procedure directly following the extension: 94°C for 90 sec followed by 30 cycles of 94°C for 30 sec, 60° C for 30 sec.

Separation of the PCR products by electrophoresis on a 12.5 % acrylamide gel.

1x CHAPS lysis buffer:

0.5 % CHAPS TRAP reaction mixture:

10 % Glycerol 20 mM Tris-HCl pH 8,3

10 mM Tris-HCl pH 7.5 1.5 mM MgCl₂

1 mM MgCl₂ 63 mM KCl

1 mM EGTA 0.05 % Tween 20

5 mM β-mercaptoethanol 1 mM EGTA

0.1 mM Benzamidine 50 µM dNTPs

	2 μ Ci	32 P-dCTP and 32 P-TTP
10X TRAP reaction buffer	0.1 μ g	TS oligonucleotide
200 mM Tris-HCl pH 8.3	0.5 μ M	T4 gene 32 protein (USB) (1 μ g)
15 mM $MgCl_2$		2 Units Taq polymerase
630 mM KCl		
0.5 % Tween 20		
10 mM EGTA		
0.1 % Bovine serum albumin (BSA)		

50X dNTP MIX = 2.5 mM each of dATP, dTTP, dGTP, dCTP

Figure 3 shows that the detection limit without radioactive labelling of the oligonucleotide for this telomerase detection method, is somewhere between 40-50 SW480 cells. The telomerase positive bands increase in strength with increasing number of cells with the exception of 100 cells sample which in this experiment did not show as much telomerase activity as expected. The manufacturer of the TRAPeze kit (Oncor, US), shows telomerase activity in an extract from 80 telomerase positive cells in their documentation.

Example 2: Isolation of telomerase activity with magnetic beads

In this experiment cultured cancer cells were combined with antibody-coated beads prior to the telomerase assay (TRAPeze kit, Oncor, US). This was a simulation of immunomagnetic separation of cells since the cell line SW480 would bind to the anti-Epithelial Dynabeads[®] through the BerEP4 antibodies on the beads. The cells captured onto the magnetic beads were lysed and the beads were separated from the lysate. Both the bead fraction and the remaining lysate were tested for telomerase activity.

Method

1000 SW480 cultured cells were washed twice in PBS, collected at 600 g for 10 minutes at 4°C, suspended in ice-cold Wash buffer, spun down again. Magnetic beads (Dynabeads[®]) of different types were used, antibody-coated Dynabeads[®] M-450 or antibody-coated Dynabeads[®] M-280. In this experiment, Dynabeads[®] anti-Epithelial Cell (M-280) was used. These beads were added to the cells and thereafter suspended in 20 μ l of ice-cold CHAPS lysis buffer. Incubation on ice for 25-30 minutes before the magnetic beads were collected at the tube wall with a Dynal MPC-E. The lysate was transferred to a new tube and the beads were suspended in 40 μ l ice-cold CHAPS lysis buffer.

For telomerase assay, a mix was prepared as in experiment 1.

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Incubation 30 minutes at 30°C for telomerase-mediated extension of the TS-primer.
Amplification procedure directly following the extension: 94°C for 90 sec followed by 30 cycles of 94°C for 30 sec, 60° C for 30 sec.
Separation of the PCR products by electrophoresis on a 12.5 % acrylamide gel.

Results

The same amount of telomerase-positive SW480 cells (1000) were lysed in the presence of magnetic beads (Dynabeads® M-280 anti-Epithelial Cell), whereas 1, 10 or 20 million magnetic beads were used. Before the TRAP assay, the beads were withdrawn from the sample using a magnet at the tube wall (DynaL MPC-E) and the lysate was transferred to a new tube. The beads were suspended in CHAPS lysis buffer. Both the bead fractions and the remaining lysates were tested for telomerase activity as shown in Figure 4. Positive telomerase signals were only seen in the bead fractions and best results were obtained using 20 million magnetic beads.

Conclusion

We conclude that the telomerase complexes surprisingly are bound directly or indirectly to the magnetic beads after cell lysis in the TRAPeze lysis buffer. When the magnetic beads were removed prior to the telomerase assay, no activity was detected (result not shown). The capture of telomerase onto magnetic beads did not destroy the biological activity of the enzyme complex. This implies that the telomerase activity can be tested by isolating the telomerase using magnetic beads and the lysate with inhibitors can be removed prior to TRAP test. Best results for capture of the telomerase complex were obtained with 20 million Dynabeads® (300µg) when using 1,000 SW480 cultured cells.

Example 3: Isolation of the telomerase complex using Dynabeads® DNA DIRECT

In example 2 telomerase was isolated using antibody-coated magnetic beads. In this example we used uncoated Dynabeads® designed for the isolation of genomic DNA directly from lysates. Variable amounts of Dynabeads® DNA DIRECT (Dyna, Oslo, Norway) were used to isolate telomerase activity from 2000 SW480 cells. The same method as in Example 2 was used.

Conclusion

The telomerase complex can be isolated from a cell lysate with uncoated magnetic beads normally used for DNA isolation. For 2000 cells the optimal amount of DNA DIRECT beads to be used for telomerase isolation is between 20 - 30 million or 300 - 450 µg.

Example 4: Isolation of telomerase with uncoated epoxide beads.

In this example uncoated Dynabeads® with epoxide polymer surface were tested for the binding of telomerase in a lysate. These beads are actually used for production of antibody-coated beads, and it was of interest to test if beads without antibodies could be used for isolating telomerase complexes. 2,000 cells were lysed in 40 µl 1x CHAPS lysis buffer with different amounts of epoxide beads (range 150-400 µg of 4.5 µm Dynabeads®). The beads were separated from the lysate after 30 minutes incubation on ice and re-suspended in 40 µl lysis buffer before the TRAP mix was added as described in Example 2.

Figure 6 shows how the signal intensity of PCR products in the TRAP assay was almost identical in the range from 200 µg to 400 µg of beads. The signal intensity in the lane with 100 µg beads was not as strong as expected, probably due to loss of cells during isolation in this experiment.

Conclusion

This example shows that all telomerase complexes were bound to the beads when 200 µg beads or more were used. Alternatively, the explanation is that enough telomerase is isolated and thereby sufficient template is produced to saturate the PCR, when using 200 µg magnetic beads or more. Cells with relative high telomerase activity were used, and as a rule of thumb a minimum of approximately 0.1 µg of epoxide Dynabeads® must be used per cell, to ensure that all or sufficient amount of telomerase complexes are isolated for strong TRAP assay PCR signals. 400 µg magnetic beads was used in later experiments.

Example 5: Telomerase isolated indirectly through binding to DNA?

The original hypothesis for the mechanism for binding of telomerase complex to the solid phase, was an indirect binding via chromosomal DNA, i.e. that the beads bound genomic DNA in a chromatin form with the telomerase complex as a part of the chromatin structure.

To test this hypothesis, the eluted material from the magnetic beads and purified material from the lysate fraction were tested for the presence of genomic DNA by PCR amplification.

Methods

The protocol in example 2 for the isolation of telomerase complexes was used. The uncoated epoxide Dynabeads® was used for this example in the same way as in example 4. Different amounts of SW480 cells were tested. Each bead fraction was resuspended in 40 µl water and incubated 5 minutes at 65° for an elution of components bound to the magnetic beads. For the remaining lysates, any genomic DNA was isolated using the commercial kit QIAamp Blood Kit from Qiagen, Germany and the recommended protocol.

Results

Testing of the bead fractions (Figure 7) showed that no PCR product could be generated for GAPDH gene in a series using 100 to 100,000 cells as starting material. However, when material from the remaining lysates were tested, positive PCR signals were seen for all samples.

Conclusion

Lack of signals in the lanes where the bead fractions were tested, and positive signals where the remaining lysates were tested, should prove that chromosomal DNA does not bind to the beads, but remains in the lysate. These results show that the telomerase complex is not isolated via binding of chromosomal DNA to the beads. The proposed hypothesis about indirect binding of telomerase through chromosomal DNA was therefore rejected.

Example 6: Mechanism for solid-phase telomerase isolation: non-specific binding of proteins and protein-RNA complexes to the magnetic beads.

Isolation of telomerase via non-specific binding of proteins or protein-RNA complexes to the beads was the next hypothesis put forward to explain the mechanism for how the telomerase can be isolated with magnetic beads. The telomerase complex was isolated following the protocol described in Example 2, using SW480 cells as starting material. The proteins were separated and visualised using SDS-PAGE with subsequent silver staining after heat elution from the beads.

Method

For analysis of the protein binding to the magnetic beads, the following protocol was used: 300 µg Dynabeads® anti-Epithelial Cell, resuspended in PBS, was added to a cell suspension with SW480 cells. The total volume was adjusted to 1 ml with PBS containing 0.1% BSA, and left on a roller for about 15 minutes at 4°C for complete mixing to occur.

The tube was placed in a MPC for 2 minutes for the bead/cell complexes to migrate to the tube wall. The supernatant was removed and discarded.

The bead-bound components were eluted from the beads by placing the tube on 65°C for 5 minutes. The tube was placed in the magnet again to collect the beads and the eluate was transferred. The tube was removed from the MPC, 1 ml PBS was added and the complex of beads and cells was resuspended. The beads with cells were collected again with the magnet and the washing was repeated twice. The resuspended complex of beads and cells was transferred to a clean tube before placing it in the MPC for the last washing.

100 µg non-coated epoxide beads was mixed in 40 µl 1x CHAPS lysis buffer and thereafter mixed with the complex of beads and cells by pipetting. The tube was incubated 30 minutes on ice for complete lysis.

The beads were collected with the magnet and the bead fraction was suspended in 10 µl 10mM Tris-HCl to a clean tube.

The PhastSystem (Pharmacia Biotech. AB, Sweden) is a SDS-PAGE system for molecular weight measurements and analysis of polypeptides. Before gel loading, 2 µl of 5x Red buffer was added to 8 µl of each of the eluates. The tubes containing this mix were placed at 100°C for 5 minutes for a denaturation of the proteins. At this step the proteins were dissociated into their respective polypeptide subunits as a result of the added SDS and β-mercaptoethanol. The tubes were stored on ice until gel loading of the samples.

The proteins were separated on a 10-15 % gradient gel by electrophoresis at a constant voltage and constant pH.

The PhastGel Silver Kit (Pharmacia Biotech. AB, Sweden) was used for visualisation of the proteins on the gel. With silver staining proteins can be detected with a concentration down to 0.3 - 0.5 ng/µl (Heukeshoven and Dernick, 1988).

The proteins bind roughly the same amount of sodium dodecyl sulphate (SDS) per weight unit and take on a net negative charge, rendering the intrinsic charge of the protein insignificant. In addition, the native conformation of a protein is altered when SDS is bound, and most proteins assume a similar shape, and thus a similar ratio of charge to mass. Separation will therefore depend on the molecular weight of the polypeptides alone. The mobility of the proteins is relatively unaffected by the polyacrylamide gel when the electrophoresis starts. When the proteins reach the gradient gel interface, their mobility is drastically reduced due to the sudden decrease in pore size, and the moving boundary rapidly migrates away from the proteins. The proteins unstack and migrate at a uniform voltage and at constant pH. As the proteins migrate through the gradient gel (10-15%), they separate according to their size.

Results

This experiment confirmed the fact that proteins bind to the epoxide beads, which is probably due to weak positive charges on the bead surface. There is yet not any known antibodies against the catalytic telomerase component hTERT, so it was not possible to conclude that hTERT was present. The protein component (hTERT) of the telomerase has a molecular weight of 127 kDa (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997). Figure 8 shows proteins of this size, so the results support the hypothesis that hTERT is one of the proteins bound to the beads. The most likely conclusion of this experiment is therefore that the protein-RNA telomerase complex binds non-specific to the magnetic beads.

Example 7: Sensitivity and reliability of the solid-phase telomerase isolation method

This example shows how reliable the method is even when using only a small quantity of cells in the sample. The protocol for isolation of telomerase (Example 2) was followed, 50 cells were used in each parallel, as this was close to the detection limit in the method developed (Example 1).

Conclusion

Even with as few as 50 cells in the sample, all five samples gave positive results for telomerase activity. This should show that both the sensitivity and the reliability of the method developed in this study is good.

Example 8: Detection of telomerase activity in micrometastatic cancer cells

To measure telomerase activity in target cells without interference of background cells like stem cells or lymphocytes in blood or bone marrow, the solution is to sort the cells prior to telomerase measurement. This can be done by isolating the cells of interest for example by using monoclonal antibodies towards cell surface markers like the BerEP4 against a common epitope of epithelial cells. Alternatively, cells that can interfere with the results can be removed by depletion for example removing T-lymphocytes by using magnetic beads coated with antibodies towards CD2. Very clean cell populations can be obtained by using a combination of positive selection or target cells and depletion of unwanted cells (Neurauter, A. et al. 1998).

In this example we show the use of cancer cell enrichment combined with telomerase isolation and subsequent TRAP assay. Cancer cells in blood will be at a very low number compared with the nucleated leukocytes in blood, down to 1 cancer cell per million normal cells or less. To obtain this assay sensitivity, a positive selection of cancer cells can be employed by taking advantage of the fact that the cancer cells are at an «unnatural» site. Carcinoma is the major group of cancers and originates from epithelial cells. Epithelial cells, even cancerous types, express certain common epithelial cell markers. Antibodies towards such epithelial cell markers (e.g. BerEP4) can be used to isolate/enrich epithelial cells by coupling such antibodies to a solid phase like magnetic beads (Hardingham et al. 1993, 1995, Borgnes et al. 1996, Naume et al. 1997). We have obtained a 3 log enrichment of the cancer cells with a recovery of about 70 % (Borgnes et al. 1996, Naume et al. 1997).

In peripheral blood cells (PBL) tested, none of 124 samples of 1000 cells tested positive for telomerase. However, using 10,000 cells or 100,000 cells many samples tested positive, 55 of 124 and 100 of 124 respectively (Shay and Wright, 1996). This implies that by removing 99.9 % of the normal cells in a blood sample this background problem can be eliminated.

Method

Known amounts of cultured cells (SW480) were washed twice in PBS, pelleted at 600 g for 10 minutes at 4° C, re-suspended in ice-cold Wash buffer, pelleted again. SW480 cells were spiked into 5 ml blood samples (around 20 million nucleated leukocytes).

20 million Dynabeads® anti-Epithelial Cell (300 µg) were added to the samples (spiked and non-spiked blood samples) and continuously mixed for 30 minutes at 4°C on a roller mixer. Captured cells were collected by using a magnet (Dyna MPC-Q) and washed 3 times in PBS. 25 µl CHAPS lysis buffer with 60µg Dynabeads® DNA DIRECT (4 million 2.8 µm beads) was added to the magnetic bead fraction with captured cells. 25-30 min incubation on ice before all magnetic beads were collected at the tube wall with an MPC-E. The lysate was discarded and the beads were re-suspended in 40 µl ice-cold CHAPS lysis buffer.

For telomerase assay a mixture was prepared as described in example 1.

Incubation 30 minutes at 30°C for telomerase-mediated extension of the TS-primer.

Amplification procedure directly following the extension: 30 cycles of 94°C for 30 sec, 60° C for 30 sec.

Separation of the PCR products by electrophoresis on a 12.5 % acrylamide gel.

Results

The immobilised cancer cells were lysed in the CHAPS lysis buffer and collecting the beads with a magnet isolated the telomerase complexes. In this example we used a combination of immunomagnetic beads and uncoated beads. The lysate was removed and in all tested cases found to be negative. Therefore, routinely we only tested the bead fractions as described in Example 2. No activity was seen in normal blood samples (unspiked) were we had followed our protocol for isolating circulating cancer cells with magnetic beads (Figure 10, lane 1). A weak signal proving telomerase activity was seen in a blood sample with 40 carcinoma cells (lane 2). Strong signals were seen in the samples with 50 to 500 carcinoma cells (lane 3-6). Positive and negative controls were as expected (lane 7-8). Lack of signals in lane 1 shows that other cells with telomerase activity, like stem cells and lymphocytes are sufficiently removed when using this method for enrichment of carcinoma cells.

Conclusion

We have demonstrated an assay sensitivity of 10 cancer cells per ml of blood or 2-3 cancer cells per million normal cells. This is the same sensitivity as shown for micrometastasis detection using immunomagnetic enrichment combined with nested RT-PCR of cytokeratin 19 mRNA (Borgnes et al. 1996). The latter assay proves the presence of epithelial cells in blood but does not indicate if this is due to cancer cells or normal epithelial cells nor does the test have any prognostic value. The advantages of using the telomerase assay are both a diagnostic and probably a prognostic value and that nested PCR can be avoided which is known to be problematic with respect to risk of contamination.

Example 9: Detection of micrometastatic cells in pleura.

All the examples so far were performed on a model system where different method parameters were examined. It was therefore very exciting when the method developed was tested on samples from breast cancer patients.

Method

There were 6 million lymphocytes, which also weakly express telomerase (Blasco *et al.*, 1995; Hiyama, K. *et al.*, 1995; Chin *et al.*, 1996), in the 12.5 ml pleura sample tested in the experiment shown here. It was likely that the patient had circulating cancer cells in the pleura (personal communication, Dr. Gunnar Kvalheim), but the quantity of cancer cells was unknown. 20 million Dynabeads® anti-Epithelial Cell were added for enrichment of cancer cells as in Example 8. The remaining cells in the pleura were lysed in 1x CHAPS lysis buffer with 400 µg epoxide beads to reveal presence of the telomerase expressing lymphocytes cancer cells not captured by Dynabeads® anti-Epithelial Cell. The protocol described in Example 2 and 8 was followed.

Results

Figure 11 shows that telomerase was detected in the enriched cell population of pleura. There seems to be some activity in the lysate fraction of this enriched population as well, which may be an indication that the number of beads added was too small for this strong telomerase activity.

Conclusion

This experiment should show that the method for isolation of carcinoma cells with subsequent detection of telomerase activity is functional when testing on a real sample as well as when tested in the model system.

Example 10: Combination of telomerase assay and CK19 assay for micrometastasis detection

The technology presently used for detection of circulating micrometastasis is based on microscopic detection of cancer cells. This can be made easier by selective staining of cancer cells made possible by cells present at an unnormal location. An example of this is staining of cytokeratin in carcinoma cells found in blood or bone marrow. Another possibility is isolation of mRNA with subsequent amplification of genes expressed more or less specifically in cancer cells.

Both the microscopic approach and the mRNA approach can be made feasible by an enrichment of cancer cells, either by depletion of normal cells or positive selection of cancer cells (Hardingham *et al.*, 1993, 1995; Eaton *et al.*, 1997; Naume *et al.*, 1997; Lycke *et al.*, 1997). However, all these strategies are more or less based on the ability to detect the cancer cell at an "unnormal" location, for example epithelial-derived carcinoma cells in blood or bone marrow.

Cytokeratin 19 (CK-19) is one of the markers used for detection of micrometastasis using the mRNA approach. The combination of immunomagnetic enrichment of circulating cancer cells with mRNA isolation and nested RT-PCR of this transcript, makes this a sensitive method with a high degree of specificity.

TRAP assay can be used for detection of the enzymatic activity of telomerase (Example 1), which is one of the most specific cancer markers known. Discovering that magnetic polymer beads can be used for an isolation of the intact telomerase complex as well as enrichment of circulating carcinoma (Example 2 and 8) improved the method for telomerase detection. In this way it will be easier both to remove factors that can inhibit the telomerase assay.

There is a problem with the diagnostic use of RT-PCR due to the possibility of false positive results. Without immunomagnetic enrichment of cells, some of the molecular markers used for detection of micrometastatic cells, e.g. CK-19, have been shown to be present in the normal samples. However, false positives may cause problems even though cancer cells are enriched from the blood sample. Normal epithelial cells may be present due to the needle's penetration of the skin when collecting blood or bone marrow. When adding immunomagnetic beads, these normal cells will be selected for as well as circulating cancer cells of epithelial origin. When using a mRNA transcript that is not specific for cancer cells, such as CK-19, a RT-PCR amplification of it can give a positive result if the blood sample is contaminated with normal epithelial cells, even though there are no circulating carcinoma present.

Table B shows a comparison of the methods most frequently used for detection of micrometastasis. All methods are sensitive, but detection based on measurement of telomerase activity is the only method that requires living cells.

Table B

Methods for detection of micrometastasis			
	Immunocytology	RT-PCR	Telomerase
Sensitivity	High	1:10 ⁶ cells	2:10 ⁶ cells
Living cells?	Not necessarily	Not necessarily	Yes
Major drawback	Time-consuming	False positives	False negatives

Expression of telomerase activity is a biological phenomenon restricted to certain stem cells (Wright *et al.*, 1996; Härle-Bachor *et al.*, 1996; Ramirez *et al.*, 1997; Kyo *et al.*, 1997; Burger *et al.*, 1997), haemopoietic cells (Broccoli *et al.*, 1995; Hiyama, K. *et al.*, 1995) and tumour cells (Kim *et al.*, 1994; Shay and Bacchetti, 1997). This specific expression pattern can be utilised to detect circulating carcinoma without considering contamination of normal telomerase negative epithelial cells. When using immunomagnetic enrichment of cells, contamination of telomerase expressing leukocytes will also be avoided.

The fact that the TRAP assay measures an enzyme activity implies that it is dependent on the presence of intact cells expressing this enzyme. Thus, the TRAP assay may be a good indicator of viable as opposed to dead tumour cells. This is important because the medical

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However, a combination of the mRNA and the telomerase approaches, as illustrated in Figure 12, would improve the reliability when detecting micrometastatic cells. Figure 12 shows the combination of the telomerase isolation with the TRAP assay and mRNA isolation from the lysate, with CK-19 as a marker. The bead traction can be used as usual for detection of telomerase activity with removal of interfering agents prior to the TRAP assay. Oligo-dT beads can be added to the lysate for isolation of polyadenylated mRNA using primers specific for CK-19 for detection of circulating cancer cells. In this way two independent tests, both very sensitive, can be performed on the same sample to diagnose micrometastasis with a high degree of reliability.

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Example 11:**Isolation of telomerase complex combined with amplification of telomerase RNA**

The RNA component has been cloned for the human template gene (Feng et al. 1995). Expression of the telomerase RNA is not correlated with telomerase activity, although expression is shown to be upregulated in cells that are highly positive for telomerase (Aylon et al. 1996, Blasco et al. 1996). The presence of telomerase RNA in cells with no telomerase activity may indicate a surplus of RNA component or inactivated telomerase complexes. The use of the RNA component for diagnosis is therefore not straightforward and presently not thought to be predictive of cancer. Telomerase RNA is not polyadenylated in humans and traditional method of total RNA isolation has been used for detection of this component.

With the method described in this invention we believe that the RNA component can be used as a diagnostic tool. The telomerase complex can be isolated by the magnetic beads and thereby the RNA component that is integrated in the intact complexes. However, free RNA component will not be isolated with this method. By combining telomerase complex isolation

and the amplification of the RNA component by RT-PCR, we believe that positive signals will correlate with telomerase activity.

Method:

Sample preparation of cells with or without the use of magnetic beads.

Cell lysis in a CHAPS lysis buffer or equivalent buffer with magnetic beads present.

Collection of the telomerase complexes immobilised to the magnetic beads using a magnet (Dyna MPC).

Removal of the remaining lysate and the bead fraction may be washed several times prior to RT-PCR of the RNA component. The RNA component may be isolated from the complex by RNA isolation procedures prior to the RT-PCR.

RT-PCR of the telomerase RNA component hTR using primers:

5'-TCTAACCCTAACTGAGAAGGGCGTAG-3'

5'-GTTTGCTCTAGAATGAACGGTGGAAG-3'

Cycles 94°C for 45 sec, 55°C for 45 sec, 72°C for 90 sec.

050907567 040202

Human telomerase RNA:

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10      20      30      40      50      60      70
.....|.....|.....|.....|.....|.....|.....|
GGGUUGCGGAGGGUGGGCCUGGGAGGGGUGGUGGCCAUUUUUUGCUAACCCUAACUGAGAAGGGCGUAG 70
GCGCCGUGCUUUUGCUCCCCGCGCGCUGUUUUUCUCGCUGACUUUCAGCGGGCGGAAAAGCCUCGGCCUG 140
CCGCCUUCCACCGUCAUUCUAGAGCAAACAAAAAUGUCAGCUGCUGGCCCGUUCGCCUCCCGGGGACC 210
UGCGGCGGGUGCGCCUGCCCAGCCCCGAACCCGCCUGGAGCCGCGGUCGGCCCGGGGCUUCUCCGGAGG 280
CACCCACUGCCACCGCGAAGAGUUGGGCUCUGUCAGCCGCGGGUCUCUCGGGGGCGAGGGCGAGGUUCAC 350
CGUUUCAGGCCGAGGAAGAGGAACGGAGCGAGUCCCGCCGCGGCGGAUUCCCUGAGCUGUGGGACGUG 420
CACCCAGGACUCGGCUCACACAUGCAGUUCGCUUCCUGUUGGUGGGGGGAACGCCGAUCGUGCGCAUCC 490
      *3' end

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PCR-primers underlined

Example 12: Isolation of colonic cells from faeces combined with telomerase assay

Telomerase activity was detected in luminal washings from colectomy specimens from 60% of tested carcinoma cases but not in cases of inflammatory bowel diseases, suggesting that it can be a good marker for the detection of colon carcinoma (Yoshida et al. 1997a). However, there is a lack of non-invasive method to make this a very useful diagnostic test. Recently a method for isolating cells from stool has been developed and patented (O'Neill 1995). The method comprises steps of a) cooling the stool to a temperature below its gel freezing point and b) removing cells using immunomagnetic beads whilst the stool remains substantially intact. Alternatively exfoliated cells can be captured from colon washings also by a immunomagnetic method. The immunomagnetically isolated cells are lysed in the presence of excess magnetic beads to capture the telomerase complexes. By using a magnet the telomerase complexes are concentrated and the rest of the lysate is discarded. In this way the telomerase activity can be measured by TRAP assay or by amplifying the RNA component. One advantage of this method is that any inhibiting substances from the stool sample can be removed prior to telomerase measurement.

The stool sample is excreted directly into a collection bag which is closed and placed in ice-water. The cooling is performed for at least 30 min.

50 ml of ice-cold aqueous suspending solution pH 7.4 comprising minimum essential Eagle' medium (MEM) is added to the stool. The bag is then agitated gently for 5 mins in ice water to wash the stool surface. The buffer should contain 50mM N-acetylcysteine, 3mM sodium butyrate, antibiotics, sodium bicarbonate (1g/litre) and BSA (10g/l). The N-acetylcysteine should be prepared fresh. Smaller samples of the stool could be used and proportionally less buffer.

The washing solution containing the suspension of cells is removed from the bag, 0.4 gram of boric acid is added per ml of washing solution to adjust the pH.

The washings are centrifuged at 250g for 10 min at 4°C and the supernatant is carefully discarded. The pellet is resuspended in 1/10 volume of ice cold suspending solution (as above).

5-10 million Dynabeads® BcrEP4 (4.5 µM) are added per ml of cell suspension. The beads may be added suspended in PBS.

The sample is incubated on a rotator at 4°C to allow for antibody-antigen immobilisation of the cells to the magnetic beads.

The immobilised cells are collected at the tube wall with a magnet (Dynal MPC) and the supernatant is removed and discarded.

The cell-bead complexes are washed in PBS by gentle stirring for 2 minutes, collected with a magnet and the washing solution is discarded.

The washing step must be repeated several times until no brown colour is seen in the washing solution.

The immobilised cells are lysed in a CHAPS lysis buffer (example 1) in the presence of sufficient amount of beads. Usually the amount is doubled by addition of DNA DIRECT magnetic beads.

The beads with any telomerase complexes are collected at the tube wall with a magnet. The isolated material is ready for telomerase assaying.

Example 13: Isolation of epithelial cells from urine and sputum/buccal swabs combined with telomerase isolation and telomerase assay.

By analysing exfoliated cells in urine telomerase activity was detected in 62% of bladder carcinoma patients, whereas weak signals were seen in 3 of 83 non-malignant urine samples (Yoshida et al. 1997b). Exfoliated cells were collected from 50 ml urine by centrifugation. The method described here uses immunomagnetic beads coated with antibodies towards epithelial cells to collect exfoliated cells from urine, sputum or other body fluids. The advantage of this is the removal of interfering substances like bacteria and debris. The telomerase complexes are isolated with magnetic beads from the cell lysate and analysed after removal of the rest of the lysate.

Urine cell sample preparation:

Urine, preferably the first urine of the day, is collected and cooled on ice.

30 million Dynabeads® BerEP4 (or other anti-epithelial antibodies) are added to the urine. The sample is rotated for 5-20 minutes to allow for cell immobilisation.

The magnetic beads with captured cells are collected with a strong magnet (Dynal MPC) and the urine carefully discarded.

The cell-bead complexes are washed twice in cold PBS before telomerase isolation.

Sputum cell sample preparation:

Saliva or sputum is collected in a bottle and diluted with ice-cold isotonic solution containing a mucolytic agent such as the suspending solution in example 5. Eating should be avoided for several hours prior to sample collection. For examination of the respiratory system the mouth should be cleaned with water prior to muco-ciliary clearance or coughing.

After ensuring that the saliva or sputum is diluted enough to reduce its viscosity, immunomagnetic beads (preferably 4.5 μ M) coated with BerEP4 are added (30 million). The sample is rotated for 5-20 minutes to allow for cell immobilisation..

The magnetic beads with captured cells are collected with a strong magnet (Dynal MPC) and the saliva/sputum carefully discarded.

The cell-bead complexes are washed twice in cold PBS before telomerase isolation.

Telomerase isolation:

The immobilised cells are lysed in a CHAPS lysis buffer (example 1) in the presence of sufficient amount of beads. Usually the amount is doubled by addition of DNA DIRECT magnetic beads.

The beads with any telomerase complexes are collected at the tube wall with a magnet. The isolated material is ready for telomerase assaying.

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KEY TO THE FIGURES

Figure 3

Lane 1: control with no cells, lane 2: 40 cells, lane 3: 50 cells, lane 4: 75 cells, lane 5: 100 cells, lane 6: 500 cells, lane 7: positive control, lane 8: negative PCR control.

Figure 4

Lane L: molecular weight ladder (10 bp)
Lane 1: lysate fraction - using 1 million Dynabeads® (15 µg)
Lane 2: bead fraction - using 1 million Dynabeads®
Lane 3: lysate fraction - using 10 million Dynabeads® (150 µg)
Lane 4: bead fraction - using 10 million Dynabeads®
Lane 5: lysate fraction - using 20 million Dynabeads® (300 µg)
Lane 6: bead fraction - using 20 million Dynabeads®

Lane 7: Lysate of 1000 SW480 cells - tested without fractionation

Lane 8: 2 µl TSR8 positive control from the kit

Figure 5

Lane 1: lysate fraction - using 10 million Dynabeads® (150 µg)

Lane 2: bead fraction - using 10 million Dynabeads®

Lane 3: lysate fraction - using 20 million Dynabeads® (300 µg)

Lane 4: bead fraction - using 20 million Dynabeads®

Lane 5: lysate fraction - using 30 million Dynabeads® (450 µg)

Lane 6: bead fraction - using 30 million Dynabeads®

Lane 7: Negative PCR control

Lane 8: 2 µl TSR8 positive control from the kit

Figure 6

Lane 1: 50 µg beads, lane 2: 100 µg beads, lane 3: 150 µg beads, lane 4: 200 µg beads, lane 5: 250 µg beads, lane 6: 300 µg beads, lane 7: 350 µg beads, lane 8: 400 µg beads and lane 9: positive TRAP assay control. And lane 10: negative PCR control.

Figure 7

Lane 1+2: 10,000 cells and the eluate of the bead bound material, lane 3+4: 5000 cells - the eluate of the bead-bound material, lane 5+6: 1,000 cells - eluate of the bead-bound material, lane 7+8: DNA isolated from the lysate of 10,000 cells, Lane 9+10: DNA isolated from the lysate of 5,000 cells, lane 11+12: DNA isolated from lysate of 1,000 cells, lane 13: positive control - 10ng placenta DNA, lane 14: negative PCR control, L: Molecular weight standard - 100 bp ladder.

Figure 8

Lane H: High molecular weight standard; Lane L: Low molecular weight standard; Lane 1&2: Eluate from beads (extract from 80 000 cells); Lane 3&4: Eluate from beads (extract from 53 000 cells).

Figure 9

Lanes 1-5: 50 cells, Lane 6: positive control, Lane 7: negative control.

Figure 10 Lane 1: 0 cells; Lane 2: 40 cells; Lane 3: 50 cells; Lane 4: 75 cells; Lane 5: 100 cells; Lane 6: 500 cells; Lane 7: positive control (500 cells directly tested); Lane 8: negative control.

Figure 11 Lane 1: Pleura sample (bead-isolated telomerase); Lane 2: Pleura sample (remaining lysate); Lane 3: Remainder of pleura sample (eluate); Lane 4: Remainder of pleura sample (lysate); Lane 5: Positive control (TSR8 template); Lane 6: Negative control.